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14. ABSTRACT Prospective epidemiological studies indicate that obesity increases the risk for prostate cancer. Also, mortality from prostate cancer is increased with elevated body weights and obesity recently was reported to be associated with higher prostate cancer grade at diagnosis and with higher recurrence rates. However, it is difficult in human studies to adequately assess effects of body weight or the effect of body weight change at specific ages on prostate cancer. Here we used the TRAMP mouse model of prostate cancer and induced obesity by injections of gold-thioglucose (GTG) at either 6, 16 or 26 weeks of age. Mice were followed until 46 weeks of age. Overall GTG led to a high death rate and obesity did not appear to negatively impact prostate cancer development or metastases. Tissue and serum analyses are now underway to evaluate adiponectin and leptin pathways. Additionally, we have initiated a study using mice with diet-induced obesity to assess the effect of body weight on tumor progression from TRAMP-C2 cells which were derived from a TRAMP prostate tumor. Presently tumor growth is being monitored.					
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INTRODUCTION:

A number of epidemiological studies indicate that increased body weight plays a role in the development of prostate cancer [1-9]. Although, not all studies have found obesity to be associated with increased risk of prostate cancer, Bergström *et al* concluded that based on obtained relative risk values 5,000 new cases of prostate cancer per year in Europe could be attributed to obesity [10]. In addition, mortality from prostate cancer is increased with elevated body weights [11], and obesity was recently reported to be associated with higher prostate cancer grade at diagnosis, as well as with higher recurrence rates [12]. The potential role of body weight in the development and progression of prostate cancer is of interest given that the incidence of overweight/obesity is increasing throughout the world, and the potential for lifestyle changes to alter body weight status. Interestingly, since we originally submitted this proposal an increasing number of publications have addressed the issue of obesity and its association with prostate cancer prognosis.

The initial goal of the present study was to evaluate the effects of obesity initiated at different ages on prostate cancer development using the TRAMP mouse model of prostate cancer. Obesity was to be induced in mice at different ages (6, 16 and 26 weeks of age) using gold-thioglucose (GTG) injections. Results of that study will be presented as well as the information pertaining to a recently initiated study.

BODY:

Although we previously used diet-induced obesity to determine effects of body weight on the development of mammary tumors, due to the variable response of mice to this intervention, *i.e.*, a range of body weights obtained, we decided to use an alternative approach to increase body weight. Our rationale was that due to the short time for development of prostate cancer in TRAMP mice and the transition of the disease through different stages, a more uniformly developing obesity model would make it easier to interpret results and fewer experimental animals would be needed. An alternative to diet-induced obesity is to damage the hypothalamus [13]. This can be done by physical/mechanical means (knife cuts and electrical lesions) or less invasively by chemical damage. Specifically, a single injection with gold-thioglucose (GTG) results in the majority of the mice gaining weight and becoming obese [14]. It is unclear why not all the treated animals respond, because when they are re-injected they then develop obesity. This is an important observation as it indicates that the initial lack of response is not due to resistance to GTG. Shortly after leptin was identified, GTG-induced obesity was reported to increase plasma leptin as identified by immunoblot; and leptin mRNA expression in adipose tissue was elevated compared to lean animals [15]. More recently when leptin levels were assessed by commercially available radioimmunoassay kits, GTG obese mice were found to have serum leptin levels two-fold higher than in control mice [16;17]. Body weight gain without consumption of a high-fat diet is obtained, although food intake is initially increased in GTG-treated mice [16;18]. Body weights eventually plateau and caloric intakes are appropriate for body weights. There is no age-sensitive time-point at which GTG needs to be administered in order to produce the effect on body weight. For example, studies have reported results for mice that were injected as young as 3-4 wks of age [19-22], while in others they were 8-12 [16;18;23;24] or 20

weeks of age [14;16;23;24]. In the literature different GTG doses over a range from 0.3-2.0 mg/g body weight have been used. In our own study in female nude mice we found that a dose of 0.5 mg/g resulted in a high mortality rate therefore we decided to undertake a preliminary study in male mice prior to injecting the TRAMP mice with GTG.

Pilot study 1

Gold thioglucose (GTG) was injected into 14 male wild-type mice at a dose of 0.5 mg/g body weight. A control group of six male wild-type mice was injected with the same volume of PBS. One mouse became ill after receiving GTG and euthanization was necessary. The mice, ranging in age from 8 to 23 weeks, were then followed for ten weeks to monitor body weight changes and general body condition. Fifty-four percent (7 out of 13) became obese. As shown in Figure 1, the GTG obese cohort gained significantly more weight than either the GTG non-obese or the control mice. There was no significant difference in the 10-week weight gain between the non-obese and the PBS injected mice. In comparison to our earlier study in female mice the 0.5 dose was well tolerated.

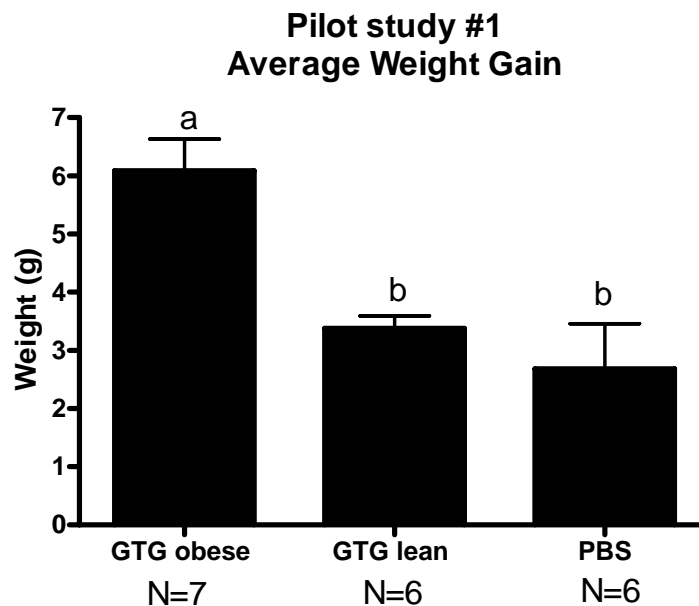


Figure 1: 10-week weight gain during pilot study 1. ANOVA $P = 0.001$; GTG obese versus GTG lean $P < 0.01$; GTG obese versus PBS $P < 0.01$; GTG lean versus PBS $P > 0.05$.

Pilot study 2

Since the 0.5 dose was well tolerated and in an effort to increase the percentage of mice that become obese with GTG treatment, a dose of 0.8 mg/g body weight was injected into 12 male wild-type mice. A control group was made up of five male wild-type mice that were injected with PBS. Four mice became ill after receiving GTG and euthanization was necessary. The mice, ranging in age from 11 to 13 weeks, were then

followed for ten weeks to monitor body weight changes and general body condition. Sixty-three percent (5 out of 8) became obese. As shown in Figure 2, the GTG obese cohort gained significantly more weight than either the GTG non-obese or the PBS-injected mice. There was no significant difference in the 10-week weight gain between the non-obese and the control mice.

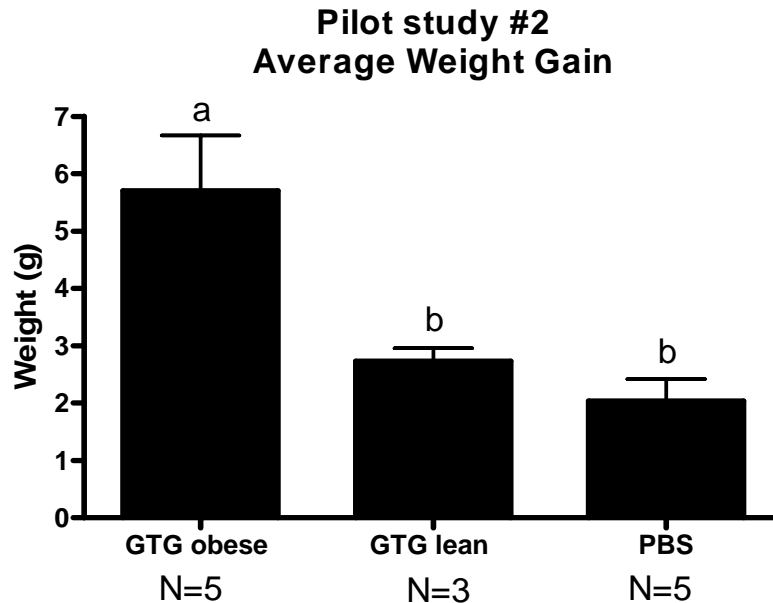


Figure 2: 10-week weight gain during pilot study 2. ANOVA $P = 0.0077$; GTG obese versus GTG non-obese $P < 0.05$; GTG obese versus PBS $P < 0.01$; GTG lean versus PBS $P > 0.05$.

Study of TRAMP mice injected with GTG at three different ages

General Methods: All mice had *ad libitum* access to purified AIN-93M diet and water. Following the pilot studies we thought that the 0.8 mg/g was the most effective dose so we proceeded with our experiment injecting mice at the three selected ages. Following GTG or PBS injections, mice were fasted for 24-hours and given free access to 2% glucose-supplemented water for one week. Body weights were recorded weekly and at that time mice were palpated for tumors. Mice that received GTG were categorized as obese or non-obese, based on weight gain relative to the PBS control mice. Serum samples were collected from the retro-orbital sinus at baseline and every 5 weeks until a tumor was palpated. Following tumor palpation, serum was collected every 3 weeks until study termination. Data are presented as mean \pm SE.

Survival: To our surprise the TRAMP mice did not tolerate the GTG as well as anticipated from the pilot studies. Several adjustments were made to the protocol in an attempt to improve survival. The dose of 0.8 mg/g was best tolerated in the mice injected at 26 weeks of age; this cohort had the highest survival at 42%. Mice in the 6- and 16-week cohorts had a much lower rate of survival at this dose, 0 and 13%, respectively. Lowering the dose to 0.5 mg/g increased survival in those injected at 6-weeks of age to

23%, but only 9% survived at this dose when injected at 16 weeks of age. A summary of the survival data is presented in Table 1.

Table 1. Percent Surviving after GTG Injection		
	0.8 mg/g	0.5 mg/g
6-week cohort	0%	23%
16-week cohort	13%	9%
26-week cohort	42%	not done

Obesity rate, weight gain and fat pad weights: After receiving GTG injections, as expected, some mice rapidly gained weight, GTG-obese, while others did not, GTG-lean. The percent that became obese was identical for the 6- and 26-week cohort at 57%. In the 16-week cohort, only 33% became obese and this lower percentage might be explained by the extremely low rate of survival. Body weight curves for the three cohorts are shown in Figure 3.. It can be seen that the GTG-lean mice and PBS control mice had similar body over the course of the study.. The actual weight gains are provided in Figure 4 where it can be seen that the GTG-lean mice gained a similar amount of weight over the experimental period as did the control mice given saline injections only. As expected the GTG-obese mice gained significantly more weight than did the two lean groups. Figure 5 presents fat pad weights. Fat pad weights were similar in the GTG-lean and control mice with values significantly lower compared to the GTG-obese groups in each age cohort.

Body Weight Curves

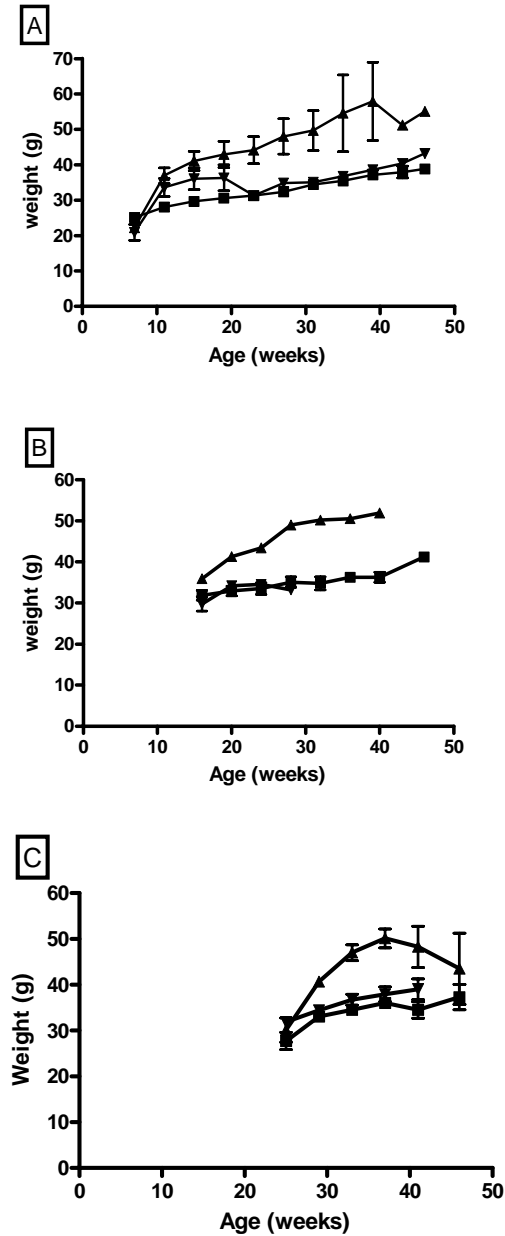


Figure 3. Body weight curves of TRAMP mice. Panel A mice injected at 6 weeks of age, GTG-obese n = 1-4; GTG-lean n= 1-3 and PBS = 2-15 dependent upon age; Panel B mice injected at 16 weeks of age, GTG=obese n=1; GTG-lean = 1-2; PBS n=4-13 dependent upon age; Panel C mice injected at 26 weeks of age, GTG-obese n=2-8; GTG-lean n= 1-6; PBS n=2.-12 dependent upon age. ▲ = GTG-obese; ▼ = GTG-lean; ■ = PBS control. For all three cohorts overall ANOVA $p < 0.001$ GTG-obese versus PBS.

Average Weight Gain

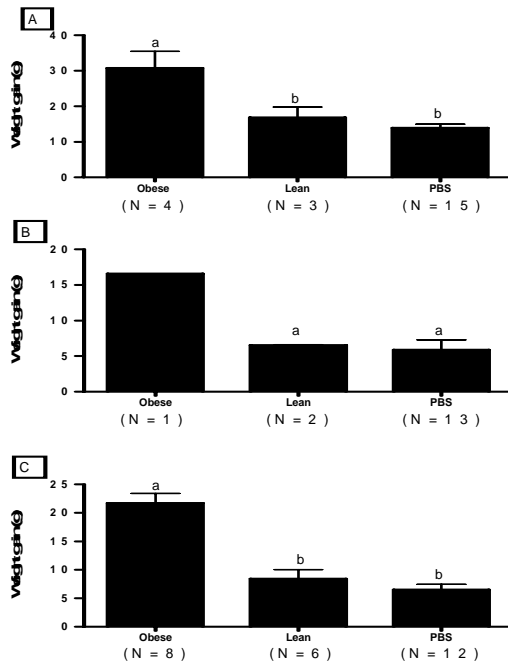


Figure 4. Average weight gain of TRAMP mice in the 6 week (Panel A), 16 week (Panel B), and 26 week (Panel C) cohorts. For both the 6 and 26 week cohorts ANOVA $p < 0.0001$ and columns with different superscripts significantly different from each other. For the 16 week cohort t test between the two lean groups was not significantly.

Fat Pad Weight

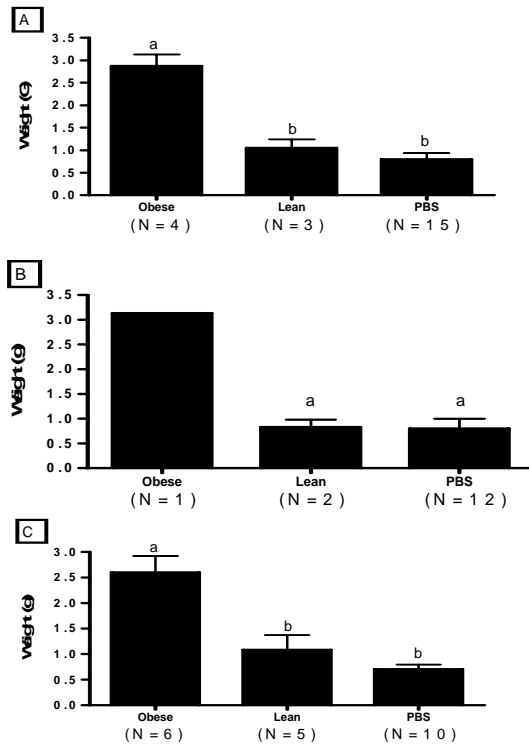


Figure 5. Average fat pad weights (sum of epididymal and retroperitoneal) for TRAMP mice in the 6 week (Panel A), 16 week (Panel B) and 26 week (Panel C) cohorts. For 6 and 26 week cohorts ANOVA $p < 0.001$, columns with different superscripts are significantly different. For 16 week cohort t test not significant between the two lean groups.

Table 2. End point comparisons for TRAMP male mice §

GTG-6	Final body weight (g)	Age at tumor palpation (weeks)	Age at death (weeks)	GU weight (g)	Tumor differentiation			Percent with metastasis
					Well	Moderate	Poor	
Obese (N=4)	51.78 ± 5.30 ^a	27.67 ± 4.91 (N=3)	35.75 ± 4.59	6.23 ± 1.40	50%	25%	25%	25%
Lean (N=3)	36.07 ± 3.75 ^b	22.0 ± 3.51	28.33 ± 8.84	6.30 ± 1.96	33%	0	67%	67%
PBS (N=15)	36.92 ± 1.33 ^b	29.36 ± 1.61 (n=14)	38.33 ± 1.92	7.84 ± 0.84	60%	7%	33%	47%
GTG-16	Final body weight (g)	Age at tumor palpation (weeks)	Age at death (weeks)	GU weight (g)	Tumor differentiation			Percent with metastasis
					Well	Moderate	Poor	
Obese (N=1)	51.9	30	40	9.42	100%			0%
Lean (N=2)	33.2 ± 1.0	25 (N=1)	24 ± 4	3.87 ± 2.4			100%	100%
PBS (N=13)	35.5 ± 1.7	26.5 ± 1.4 (N=12)	35.6 ± 2.5	7.3 ± 0.8	54%	8%	38%	46%
GTG-26	Final body weight (g)	Age at tumor palpation (weeks)	Age at death (weeks)	GU weight (g)	Tumor differentiation			Percent with metastasis
					Well	Moderate	Poor	
Obese (N=8)	48.61 ± 2.60 ^a	33.38 ± 0.94	42.13 ± 1.22	9.10 ± 1.54 (N=6)	50%	50%	0	13%
*Lean (N=6)	39.08 ± 2.46 ^b	33.5 ± 1.09	43.0 ± 1.67	8.90 ± 1.45 (N=5)	67%	17%	0	17%
PBS (N=12)	36.65 ± 1.63 ^b	32.3 ± 1.1	41.3 ± 1.4	8.16 ± 1.0 (N=11)	58%	8%	33%	33%

§ columns with different letters indicate a significant difference among the groups

*pathology report not received for one mouse in this group

Prostate cancer results: A summary of the results for the cohorts is presented in Table 2. In all three cohorts final body weights were significantly higher for the GTG-obese mice compared to the GTG-lean and PBS control mice. Overall for the other determinations there were no significant differences partially attributable to the small sample sizes in GTG-obese and GTG-lean groups. However, a few interesting observations were made. For example, the GTG-obese mice injected at 6 weeks of age had a delay in tumor detection compared to the GTG-lean mice and a delayed age at death. Genital-urinary tract (GUT) weight was not impacted by body weight. Tumor differentiation was improved and metastases rate was reduced in GTG-obese mice compared to the PBS control mice. Due to the poor survival rate for the mice injected at 16 weeks of age it is not possible to make any conclusions for this age group. For the mice injected at 26 weeks of age, age of tumor detection, age at death and GUT weights were similar in all three groups. There was however, a trend for the GTG-obese mice to have an improved tumor differentiation profile compared to both lean groups and to have a reduced metastasis rate compared to the control saline injected mice. We will next proceed with serum analyses of the mice to determine if adipokines or sex hormones may play a role in these findings.

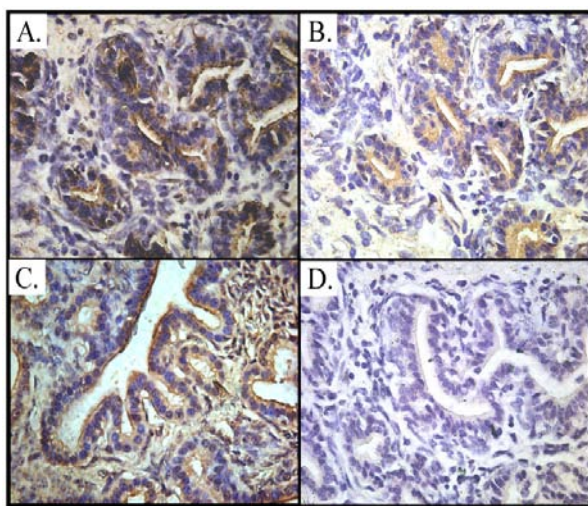
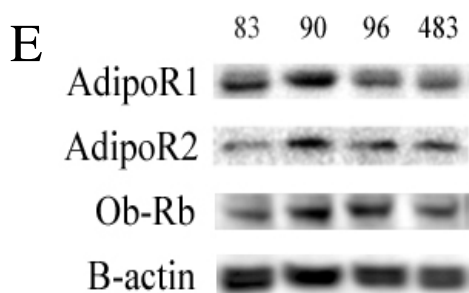


Figure 6. Expression of Acrp30 and leptin receptors from TRAMP prostate tumor tissue. Immunohistochemistry of A) AdipoR1, B) AdipoR2, C) Ob-Rb and D) control with goat serum instead of primary antibody. The presence of reddish brown color indicates a positive reaction for the specific primary antibodies. Arrows indicate areas of positive staining for each antibody. Hematoxylin was utilized for the blue counter staining of the nuclei. E) Westerns of AdipoR1, AdipoR2 and Ob-R from TRAMP prostate tumor tissues from four different TRAMP animals. The individual animal numbers at the top of the blots. The antibodies used are shown along the left hand side.



Expression of AdipoR1, AdipoR2 and Ob-Rb in tumor tissue from mouse prostate cancer: To further strengthen the relationship between Acrp30, leptin and prostate cancer growth we investigated whether tumors from TRAMP mice express the two receptors for Acrp30, AdipoR1 and AdipoR2 and the signaling form of the leptin receptor, Ob-Rb. Tumors from four different mice were examined using immunohistochemistry and western analysis. Representative staining from the immunohistochemistry is shown in Figure 6.

Expression of AdipoR1 was found in all prostate tumor tissue from TRAMP mice and was primarily located in epithelial cells on the apical region (Fig 6A). In samples from all mice AdipoR2 (Fig 6B) was present primarily in the same areas as AdipoR1 but the staining was less intense. Ob-Rb (Fig 6C) was expressed throughout the samples. Control staining with goat serum instead of primary antibody was negative (Fig 6D). Western blot analysis of frozen tissue from the same mice was also performed and expression of AdipoR1, AdipoR2 and leptin receptor was found in all prostate tumor tissues examined (Fig 6E).

Study using TRAMP-C2 Cells

To continue our goal to understand the impact of obesity on prostate cancer we are now using a different approach. We will use a prostate cancer cell line derived from a TRAMP prostate cancer tumor [25]. The cells will be implanted into C57BL6 mice which are the background strain of the TRAMP mouse. Several previous

studies have reported that inoculation of two different lines TRAMP-C1 and TRAMP-C2 resulted in tumor formation in C57BL6 mice [25;26]. The use of this cell line will provide a more straight forward approach than using athymic mice in xenograft studies of human prostate cancer cells and will allow for blood sampling during the study as the mice do not need to be maintained in an ultraclean environment. Also it is fortuitous to be able to use C57BL6 mice as these mice readily develop dietary-induced obesity. Furthermore, use of this strain provides the unique opportunity to evaluate prostate cancer cell ability to develop tumors in mice fed the same diet but with different body weights. Previous studies in our lab and in other labs have shown that although most C57BL6 mice fed a high-fat diet will gain weight and will become overweight or obese, some will stay in the body weight range of low-fat fed mice [27;28]. This occurrence provides the opportunity to compare mice of the same body weight consuming diets of different composition as well as to compare mice fed the same diet but with different body weights.

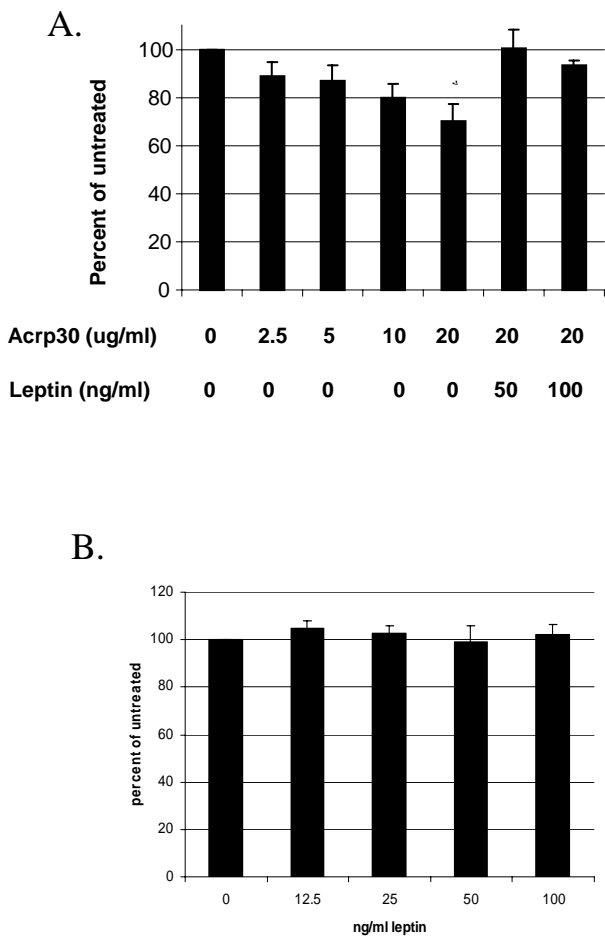


Figure 7. Proliferation of TRAMP-C2 cells 48 hours after treatment with Acrp30, leptin or both. Cell proliferation as a percent is shown along the y-axis. Cells in serum-free media were considered to be 100%. A) The concentrations of Acrp30 are shown along the x-axis. Bars represent standard error of the mean from the four different experiments and asterisks are significantly different from untreated. (ANOVA p=0.0393). The concentrations of Acrp30 and leptin are shown along the x-axis. Bars represent standard error of the mean from the different experiments. B) Cell proliferation of the TRAMP-C2 cells in response to increasing levels of leptin (ng/ml). The concentration of leptin is shown along the x-axis. Bars represent standard error of the mean from three different experiments.

In vitro growth and signaling of TRAMP-C2 cells in response to Acrp30 and leptin: In conjunction with the *in vivo* studies we investigated the effects of Acrp30, leptin or both on proliferation of the TRAMP-C2 prostate cancer cell line *in vitro*. In figure 7A the cells were treated with Acrp30 in the physiological range of 2.5-20 μ g/ml. There was a dose-related reduction in proliferation of the TRAMP-C2 cells after 48 hours (Fig 7A). The difference in proliferation was statistically significant at the Acrp30 concentration of 20 μ g/ml compared to the untreated cells. The cells had 70% proliferation compared to the untreated controls. In addition, cells were treated with a combination of Acrp30 and leptin. We found that the addition of leptin blocked the ability of Acrp30 to inhibit proliferation. However, figure 7B shows that treatment of the cells with leptin alone in its physiological range for 48 hours did not result in a statistically significant change in cell proliferation. This data strengthens the possibility that prostate cancer proliferation can be inhibited by Acrp30 and that leptin can block this protective effect.

In vivo experiments with TRAMP-C2 cells: We have obtained C57BL6 male mice (n=160) from Jackson Laboratory, Bar Harbor ME in groups of 40. From weaning at 4 weeks of age mice were maintained on AIN-93M diet (Table 3). At 6 weeks of age 120 mice were switched AIN-93M-High-Fat diet (Table 3). When

C57BL6 mice are fed high-fat diets Obesity-Prone and Obesity-Resistant groups can be identified [27]. Serum samples will be obtained at 20 weeks of age to determine serum leptin levels and mice will be stratified by body weight classification based on weight gain from 6-20 weeks of age. Based on three previous trials approximately 2/3 of the mice gain weight (2 standard deviations above controls) and are classified as Obesity-Prone and/or Overweight. The remaining high-fat diet mice are designated as Obesity-Resistant as they remain in the body weight range of the low fat (AIN-93M) diet mice. At this time the 40 heaviest mice will be assigned to the Obesity-Prone group, the middle weight mice will be assigned to Overweight and the lightest third to the Obesity-Resistant group. At this time one half of the mice in each group will be switched to the low-fat diet for the remainder of the experiment (Table 4). Additionally at 20 weeks of age half of the 40 mice maintained on the AIN-93M diet will be switched to the high-fat diet to determine the effect of the diet per se on tumor development.

Table 3. Composition of Experimental Diets

	AIN-93M ¹ (mg/g)	AIN-93M-High-Fat ² (mg/g)
Casein	140.0	190
L-Cystine	1.8	2.44
Corn starch	470.692	305.95
Maltodextrin	160.0	104
Sucrose	100.0	65
Soybean oil ³	40.0	160
Cellulose	40.0	98.4742
AIN-93-MX -Mineral mix	35.0	47.25
AIN-93-VX- Vitamin mix	10.0	13.5
Choline bitartrate	2.5	3.375
TBHQ (antioxidant)	0.008	0.0108

¹Based on AIN-93M diet designed for long-term maintenance of rodents. ²Updated high fat diet based AIN-93M diet.

³Although soybean products such as soy meal and protein are associated with anticarcinogenesis, this is not true for soybean oil. Soybean oil improves the fatty acid n-6/n-3 ratio. Corn oil used in AIN-76A diet with high n-6 fatty acid content was thought to support tumor growth. No phytoestrogens have been found in AIN-93 diet.

Table 4. Experimental Groups and Diets for TRAMP-C2 Tumor Study

Group Designation	6-20 Weeks of Age Diet Consumed	20-28 Weeks of Age Diet Consumed
Lean	Low-Fat	Low-Fat
Lean-High-fat	Low-Fat	High-Fat
Obesity-Resistant	High-Fat	High-Fat
Obesity-Resistant-Low-Fat	High-Fat	Low-Fat
Overweight	High-Fat	High-fat
Overweight-Low-Fat	High-Fat	Low-Fat
Obesity-Prone	High-Fat	High-Fat
Obesity-Prone-Low-Fat	High-Fat	Low-Fat

At 20 weeks of age at the time of obtaining blood samples all mice will be subcutaneously inoculated with TRAMP-C2 prostate cancer cell lines (2×10^6 cells) as a 200 μ L suspension in 50% Matrigel. Cells will be

grown and will be maintained in RPMI 1640 medium containing fetal bovine serum supplemented with penicillin (100 units/mL) and streptomycin (100 µL). Following cell inoculation mice will be followed for 8 additional weeks to determine age of tumor detection (latency), incidence and rate of tumor growth. Body weights will be assessed weekly and mice examined for tumor development. The age of tumor detection will be based on the ability to measure a growth of 5mm. Once tumors are identified they will be monitored twice weekly to ensure health status of the mice. Tumor length (l) and width (w) will be assessed with calipers, and tumor volume calculated ($V = 0.4 \times l \times w^2$). Mice will be euthanized for the following reasons; tumors reach 20 mm in diameter, weight loss of 20% occurs, tumors ulcerate, there are other health related issues or the mouse reaches the terminal age of 34 weeks. Three hours prior to sacrifice mice from each group will be injected with 5-bromo-2'deoxyuridine (BrdU) for determination of tumor apoptosis and proliferation rates. At euthanasia, blood will be obtained for serum leptin and adiponectin determinations. Tumor samples will be prepared for histopathological analyses, as well as lymph nodes, lungs, livers and spleens to determine metastasis rates. Urogenital tracts will be removed and weighed. Tumor weight relative to body/carcass weight will be calculated to assess tumor burden. Retroperitoneal and epididymal fat pads will be dissected and weighed and used as a surrogate of body fatness.

At the present time, all of the cohorts have reached 20 weeks of age, at which time a retro-orbital blood sample under anesthesia was obtained. All mice in these cohorts were inoculated with TRAMP-C2 cells (a subcutaneous injection of 3 million cells in 50% matrigel, on the right flank) at 20 weeks of age.

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Performed experiments as described to induce obesity at specific ages.
- 2) Preliminary interpretation indicates that obesity at a young age may be protective with respect to the development of prostate cancer. This is consistent with some human epidemiological studies.
- 3) Found that toxicity and mortality associated with GTG-induced obesity makes it impractical for continued use.
- 4) Determined that AdipoR1, AdipoR2 and Ob-Rb are expressed by TRAMP prostate cancer cells.
- 5) We are now using an alternative approach to address the issue of the effect of body weight on prostate cancer development.
- 6) Performed in vitro experiments that indicate that TRAMP-C2 cell proliferation is inhibited by Acrp30 and that this effect is blocked by high levels of leptin.

REPORTABLE OUTCOMES:

ROLE OF OBESITY AT DIFFERENT AGES IN PROSTATE CANCER DEVELOPMENT IN TRAMP MICE

Margot P. Cleary, Melissa J.L. Bonorden, Olga P. Rogozina and Nancy K. Mizuno

Presented at the IMPACT meeting September 2007, Atlanta, GA. (abstract included Appendix A)

CHARACTERIZATION OF ADIPONECTIN RECEPTOR EXPRESSION AND FUNCTION IN TRAMP PROSTATE TUMORS AND THE TRAMP-C2 CELL LINE

Michael E. Grossmann, Nancy K. Mizuno, Melissa J. L. Bonorden, Amitabha Ray and Margot P. Cleary
Hormel Institute, University of Minnesota, Austin, MN

Presented at the Frontiers in Cancer Prevention Research meeting December 2007, Philadelphia, PA (abstract included Appendix B)

ROLE OF THE ADIPONECTIN LEPTIN RATIO IN PROSTATE CANCER

Michael E. Grossmann, Nancy K. Mizuno, Melissa J.L. Bonorden, Amitabha Ray, Irina Sokolchik², Meena L. Narasimhan and Margot P. Cleary¹

Submitted (abstract included Appendix C)

CONCLUSIONS:

This has been a frustrating experience over the first two years trying to undertake what we thought was a straightforward approach to inducing obesity in the mice. At this time we hope that our new approach will provide more insightful results into the effect of obesity on prostate cancer.

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Appendix A

ROLE OF OBESITY AT DIFFERENT AGES IN PROSTATE CANCER DEVELOPMENT IN TRAMP MICE

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A number of epidemiological studies have implicated obesity as a risk factor for prostate cancer development. In addition, clinical and biochemical progression of prostate cancer has been reported to be shorter in obese men and mortality from prostate cancer is increased with elevated body weights. Obesity was recently reported to be associated with higher prostate cancer grade at diagnosis, as well as with higher recurrence rates. The potential role of body weight in various aspects of prostate tumorigenesis is of interest given that the incidence of overweight/obesity is increasing throughout the world, and the potential for lifestyle changes to alter body weight status. The goal of the present study is to determine the effect of obesity induced at different ages on the development of prostate cancer using the TRAMP mouse model. Development of prostate cancer in the TRAMP mouse shares a number of similarities with the human disease. The experimental design was to induce obesity at three different ages and follow prostate cancer development. To attain this goal male TRAMP mice (C57BL6 background) were injected with gold-thioglucose (GTG) (0.5-0.8 mg/kg body weight in phosphate buffered saline (PBS)) at 6, 16 and 26 weeks of age. Mice were weighed weekly and palpated to detect prostate tumors. Mice were followed until 46 weeks of age or until disease burden necessitated euthanasia. Serial blood samples over the course of the study are also obtained. Control mice received injections of only the PBS vehicle. For the 26-week cohort 14 mice survived the GTG injection, of which 8 were obese (48.6 ± 2.6 grams). As expected some of the GTG mice did not develop obesity and were designated as non-Obese. Their body weight 39.1 ± 2.5 was in the body weight range of the PBS mice (36.7 ± 1.63). The final body weights for the Obese mice were significantly higher than for the other two groups (ANOVA $p < 0.05$). Fat pad weights followed a similar relationship. Total genital-urinary tract weights were not affected by body weight. Age of prostate tumor detection was not different among the three groups of mice (~33 weeks of age in age). Additionally age at death (~43 weeks of age) was similar among the groups. Serum and tissue analyses are presently being conducted. Additional cohorts of mice injected with GTG at 6 and 16 weeks of age are currently being followed. Overall it appears that GTG has a high toxicity and mortality rate in TRAMP mice (in contrast to our preliminary studies in C57BL6) mice. In the older mice induction of obesity had little effect on the development of prostate cancer. Ongoing studies will address the consequence of obesity on prostate cancer development in younger TRAMP mice.

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Appendix B

Characterization of Adiponectin Receptor Expression and Function in TRAMP Prostate Tumors and the TRAMP-C2 Cell Line

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Introduction: Obesity is associated with increased risk for more aggressive prostate cancer (Pca) as defined by an increase in the risk of Pca death and an increased chance of progression after surgery. Obesity may mediate its effects on Pca in part due to factors secreted from adipose tissue. One factor potentially involved in the interaction between Pca and obesity is adiponectin, also known as adipocyte complement-related protein of 30 kDa (Acrp30). Lower serum Acrp30 levels have been reported for Pca patients compared to patients with benign prostatic hyperplasia or healthy controls. In addition, lower expression levels of Acrp30 receptors are found in prostate tumors as compared to healthy prostate tissue. Here we assessed how Acrp30 impacted cell growth *in vitro* in the TRAMP-C2 cell line which is derived from a TRAMP prostate tumor and determined Acrp30 receptor expression in the TRAMP model.

Procedures: TRAMP-C2 cells (ATCC) were used in growth assays (CC8 kit Dojindo Laboratories). Whole cell extracts were obtained using Phosphosafe extraction reagent from Novagen for determination of adiponectin receptors (AdipoR1 and R2) and signaling proteins by western blot. Antibodies were from Santa Cruz Biotechnology except antibodies to AdipoR1 (Abcam Inc), AdipoR2 (Phoenix Pharmaceuticals, Inc.) and anti-rabbit secondary (Cell Signaling Inc.). TRAMP mice were euthanized at 50 weeks and urogenital tracts plus abnormal growths/tumors removed. Sections were stained with the rabbit ABC staining system for AdipoR1 and R2.

Results: There was a dose-related reduction in proliferation of the TRAMP-C2 cells after 48 hours in response to the addition of Acrp30. The difference in proliferation was statistically significant at physiological Acrp30 concentrations of 10 and 20 ug/ml (Student's t-test $p < 0.03$ and 0.02 respectively) compared to untreated cells. Western blots indicated that AdipoR1 and AdipoR2 are both expressed by TRAMP-C2 cells. We also identified increases or decreases in phosphorylation of several growth associated signaling proteins with western blots. Acrp30 increased levels for both ERK1 and ERK2. The phosphorylation of Stat3 was decreased by the addition of fetal calf serum but this decrease was blocked by Acrp30.

We also found that tumors from TRAMP mice expressed the two receptors for Acrp30, AdipoR1 and AdipoR2. Using immunohistochemical analysis we found expression of AdipoR1 in prostate tumor tissue from TRAMP mice was mostly in epithelial cells on the apical membrane. AdipoR2 was present in the same areas as AdipoR1 but the staining was lower. Western blot analysis of frozen tissue from the same mice also indicated expression of AdipoR1 and AdipoR2 in prostate tumor tissue.

Conclusions: Here, we are the first to report the presence of AdipoR1 and AdipoR2 in prostate tumor tissues from TRAMP mice and in the TRAMP-C2 cell line which is derived from the prostate tumor of a TRAMP mouse. The receptors appear to be functional since proliferation of TRAMP-C2 cells was inhibited by addition of Acrp30. This decrease in cell growth may be attributable to increased signaling through ERK 1/2 since Acrp30 increased the phosphorylation of ERK 1/2. We are currently investigating the levels of Acrp30 *in vivo* with ongoing mouse studies in relationship to body weight and Pca development. Support from DOD PC 050284 and The Hormel Foundation.

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Appendix C

Role of the Adiponectin Leptin Ratio in Prostate Cancer

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Abstract

The effects of obesity on prostate cancer are complex and controversial. In order to better understand these effects we have investigated two different proteins that are secreted by adipose tissue. Adiponectin and leptin have been studied individually for how they affect proliferation, signaling and apoptosis of prostate cancer. However, while both are expressed by virtually all people the effect of the ratio of the two on prostate cancer initiation and progression remains to be elucidated. Transgenic adenocarcinoma mouse prostate (TRAMP) mice develop prostate cancer in a manner similar to that of humans. The TRAMP-C2 cell line was derived from a TRAMP tumor and can be utilized to investigate effects of various treatments *in vitro* or *in vivo*. Here we have investigated the effects of adiponectin, leptin or the combination of both in this model of prostate cancer. We found that tumors from TRAMP mice express both adiponectin and leptin receptors. Also, addition of adiponectin to TRAMP-C2 prostate cancer cells reduced proliferation. Leptin treatment alone did not significantly alter proliferation. However, when leptin was combined with adiponectin at ratios seen in obese individuals, leptin was able to block the ability of adiponectin to reduce cell proliferation. Adiponectin and leptin can alter signaling of the ERK pathway as shown by changes in phosphorylation. The plant protein osmotin has previously been shown to activate adiponectin receptors and we found that osmotin reduces proliferation of TRAMP-C2 cells in a manner similar to adiponectin. This work suggests that adiponectin, leptin and their associated receptors may play an important role in prostate cancer.